No inbreeding avoidance by female burying beetles regardless of whether they encounter males sequentially or simultaneously

No Inbreeding Avoidance When Females Encounter Males Simultaneously or Sequentially in the Burying Beetle *Nicrophorus vespilloides*

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Manuscript type: Article
Running title: Inbreeding Avoidance by Mate Choice

Manuscript information:
Abstract: 241 words
Main text (not including references and legends): 4,250 words
Figures: 2

Abstract

Inbreeding avoidance reduces the probability that an individual will mate with a related partner, thereby lowering the risk that it produces inbred offspring suffering from inbreeding depression. Inbreeding avoidance can occur through several mechanisms, including active mate choice, polyandry and sex-biased dispersal. Here, we focus on the role of active mate choice as a mechanism for inbreeding avoidance. Recent evidence suggests that the experimental design used in mate choice experiments (i.e., simultaneous versus sequential choice) can have a strong impact on strength of the reported mating preferences. In this study, we examine whether similar effects of experimental design also apply in the context of inbreeding avoidance. To this end, we designed two experiments on the burying beetle *Nicrophorus vespilloides* that matched two different contexts under which females encounter potential mates in the wild; that is, when females encounter males simultaneously and sequentially. We found that females were as likely to mate with related and unrelated males regardless of whether they encountered male partners simultaneously or sequentially. Thus, our study provides no evidence for inbreeding avoidance in this species, and suggests that the number of mates present did not influence the degree of inbreeding avoidance. We discuss potential explanations for the lack of inbreeding avoidance through mate choice, including lack of mechanisms for recognising close relatives, low costs and/or low risks of inbreeding and the presence of other inbreeding avoidance mechanisms, such as sex-biased dispersal and polyandry coupled with post-copulatory mate choice.

Keywords: inbreeding avoidance, *Nicrophorus vespilloides*, sequential mate choice, simultaneous mate choice.

Introduction

Inbreeding avoidance occurs when an individual exhibits traits that reduces the probability that it will mate with a related partner, thereby reducing the risk that it produces inbred offspring suffering from inbreeding depression (Blouin & Blouin 1988). Inbreeding avoidance may be based on a number of different mechanisms, including active mate choice, polyandry (including extra-pair copulations), and sex-biased dispersal (Pusey 1987; Pusey & Wolf 1996). Active mate choice based on cues about relatedness, whereby an individual (typically a female) avoids close relatives of the opposite sex as social or sexual partners, has been documented in birds, mammals, fishes, and arthropods (Fadao et al. 2000; Frommen & Bakker 2006; Gerlach & Lysiak 2006; Hansson et al. 2007; but see Szulkin et al. 2012). A theoretical model by Kokko & Ots (2006) predicts that inbreeding avoidance is more likely to evolve when mate choice is simultaneous rather than sequential. The reason for this is that the female can choose whom to mate with when potential mates are encountered simultaneously, whilst she must choose whether or not to mate when potential mates are sequential. Thus, when mates are encountered sequentially, inbreeding avoidance could be associated with a considerable cost in terms of lost breeding opportunities. The model also predicts that inbreeding avoidance is more likely to evolve in species where inbreeding depression is severe and where both sexes invest heavily in parental care towards the offspring (Kokko & Ots 2006).

We tested for inbreeding avoidance by females through simultaneous and sequential mate choice in the burying beetle, *Nicrophorus vespilloides*. This species is useful as a model for studying inbreeding avoidance because there is good evidence that inbreeding incurs a severe fitness cost to the offspring (Mattey et al. 2013), and because both sexes invest heavily in care (Smiseth & Moore 2004; Smiseth et al. 2005). These considerations suggest selection should favour inbreeding avoidance in this species (Kokko & Ots 2006). Previous work suggests that mating in this species may occur in contexts where females encounter males either simultaneously or sequentially (Eggert 1992; Muller et al. 2006).

Simultaneous encounters occur in the presence of a small vertebrate carcass, which serves as a resource for breeding (Scott 1998). Multiple males and females often locate the same carcass, resulting in the potential for simultaneous mate choice coupled with intra-sexual competition (Otronen 1988; Eggert 1992). The dominant pair will mate repeatedly and then cooperate to rear the resulting brood (Eggert & Müller 1989; Eggert & Müller 1997; House et al. 2009). In contrast, sequential encounters occur in the absence of a carcass when females approach single pheromone-emitting males (Eggert 1992). In this context, females can choose whether or not to mate with a particular male (House et al. 2008; Head et al. 2014). When females mate with a male without a carcass, they store the sperm for potential use in future breeding attempts (Bartlett 1987).

The aim of our study was to test for inbreeding avoidance when females encounter potential mates either simultaneously or sequentially. We first tested for inbreeding avoidance when mates are encountered simultaneously in the presence of a carcass by recording copulation rates of females presented with a choice between two potential mates, one related and one unrelated to the female. We next tested for inbreeding avoidance when mates are encountered sequentially in the absence of a carcass by recording successful copulations by females that were presented with a single related or unrelated male. Based on the model by Kokko & Ots (2006), we predict that females will preferentially mate with unrelated males given evidence for severe inbreeding depression and high levels of parental care by both sexes in this species. Furthermore, based on this model, we predict that females should be more likely to avoid mating with related males when females encounter males simultaneously given that inbreeding avoidance in this context is associated with a negligible cost in terms of lost breeding opportunities.

Materials and Methods

General Methodology

All beetles used in the experiments were from a large outbred laboratory population maintained at the University of Edinburgh. As a matter of routine practice, we always house all beetles in individual transparent plastic boxes (12cm x 8cm and 2cm high) from the time that they dispersed from the carcass as third instar larvae to ensure that we have full control over the pedigree of our stock population. Furthermore, when beetles are paired for breeding, we mate each female with one male to prevent multiple paternity. Thus, we know the identity of the ancestors of every beetle in our laboratory population dating back to the wild-caught beetles. Furthermore, keeping beetles in individual boxes also ensures that all experimental beetles were virgins at the start of the experiments. All beetles were maintained at 20°C under constant light, and were fed organic beef twice a week once they had become adults. The population used in both experiments comprised of beetles originally collected at Corstorphine Hill and Hermitage of the Braid Edinburgh, UK; Jodrell Bank, Manchester, UK; Kennel Vale, Cornwall, UK; and Madingley Woods, Cambridge, UK. We considered a male that was a full sibling with a given female to be closely related to the female, while a male that did not share a common grandparent or closer relative with a given female was defined as unrelated to the female. We used each female and male only once in the experiments to avoid potential effects on female and male behaviour due to prior experience. For all trials, we recorded the size of both males and females by measuring the width of the individual's pronotum using digital callipers. We did this to account for potential effects of either male or female size on the females' mating preferences. For example, a study on the closely related *N. orbicollis* found that females generally preferred mating larger males, but that this preference was dependent on the female's own body size (Beeler et al. 2002).

Simultaneous Mate Encounters

To test for inbreeding avoidance when a female encounters potential mates simultaneously, we presented a single female with two males, one of which was closely related to the female (i.e., full sibling) and one of which was unrelated to the female (i.e., did not share a common

grandparent or closer relative). In this experiment, we placed the beetles in a large transparent box (17 cm x 12 cm and 6 cm high) that was filled with 0.5 cm of moist soil and provided with a previously frozen rat carcass (supplied from Livefoods Direct Ltd, Sheffield, UK). This design was used to simulate situations in the wild where a female encounters multiple males on a carcass. We used a relatively large rat carcass (range: 41–82 g), because it allowed us to tether one male to the right front leg and the other male to the left hind leg. This procedure allowed us to prevent that competition between the two males, which commonly occurs when multiple males locate the same carcass, would restrict the female's ability to choose between the males (Otronen 1988). The female was always placed on the carcass between the two males. There was no effect of the size of the carcass on the number of copulations during trials ($Z = -0.32$, $p = 0.75$). We alternated whether it was the related or the unrelated male that was tethered to the front leg of the carcass between different trials to exclude any potential effects due to a bias towards males in different positions on the carcass. We tethered the males by tying one end of a string of dental floss around the male's pronotum and attaching the other end to the right foreleg of the carcass. We always ensured that each male was tethered such that he was able to mate with the female by providing him with 2 cm of give. At the start of the trial, the female was placed at the centre of the carcass and we recorded the time at which the female first contacted each male and the number of successful copulations that each male had with the female over the following 60 min. Successful copulations occurred when the male inserted his adeagus (intromittent organ) into the female's vagina, and each copulation lasts for about 90 s (House et al. 2008). Females mate repeatedly with the same male both on and off carcass and do not have a refractory period (House et al. 2008). In this experiment, we set up 30 trials in total, four of which were excluded from further analyses because one of the males escaped during the observation.

Sequential Mate Encounters

To test for inbreeding avoidance when a female encounters potential mates sequentially, we presented a single female with a single related or unrelated male. In this experiment, we placed the beetles in a petri dish (90mm diameter, and 12mm high) based on established protocols for studying mating off carcasses in this species (House et al. 2008; House et al. 2009; Head et al. 2014). This design was used to simulate situations in the wild where a female encounters a single male. If a mating took place, we also recorded latency to mating defined as the time it took from when the pair were placed in the Petri dish until the first mating took place. If the pair did not mate within the 30 min trial period, the pair was scored as not to have copulated successfully. In this experiment, we set up 50 experimental pairs in total.

Statistical Analyses

All statistics were carried out using R version 2.15.1. In the analysis of the simultaneous mate choice trails, we tested whether females copulated more frequently with the unrelated male than with the related male. To this end, we used a generalised linear mixed model with a Poisson error distribution, where we included the female's relatedness to the male (unrelated or related), male size, female size, whether the male was the first to come into contact with the female (yes or no), and the male's position on the carcass (front or hind leg) as fixed factors. In all models, female identity was added as a random effect to account for the non-independence between the observations on the two males in the same trial. Before using this method, we first confirmed that there was not a negative correlation between the number of times the female copulated with the two males in a trial (Spearman's rank test: $\rho = 0.125$, $n = 26$, $p = 0.544$,). Such a negative correlation would be expected if mating with one male was mutually exclusive with mating with the other male. There were no significant effects of male size or the interaction between male size and relatedness on the number of times the female copulated with each male ($Z = 0.44$, $p = 0.73$ and $Z = -1.4$, $p = 0.16$, respectively), and these terms were therefore not included in the final model. In the analysis of the sequential mate choice trials, we first tested whether copulations were more likely to

be successful when a female was provided with an unrelated male than when she was provided with a related male. To this end, we used a generalised linear model with a binomial error distribution, which included the female's relatedness to the male (related or unrelated), male size and female size as factors. Male size and female size had no significant effect on whether the pair copulated successfully or not (Z_{47} = -1.53, p = 0.13 and Z_{47} = -1.48, p = 0.14, respectively), and these terms were therefore not included in the final model. For those pairs that successfully copulated, we then tested whether a female took longer to copulate when presented with a related male as compared to when she was presented with an unrelated male. To this end, we used a generalised linear model with a quasi-Poisson error distribution (to account for over-dispersion of the data), which included the female's relatedness to the male (unrelated or related), male size and female size as fixed factors. There was no effect of either male size or female size on the latency to copulate (Z_{38} = -1.68, p = 0.1 and Z_{38} = -1.89, p = 0.067, respectively), and these terms were therefore excluded from the final model.

Results

Simultaneous Mate Encounters

Females copulated successfully with only one of the two males in 11 out of 26 trials. Females mated with the unrelated male in 2 of these trials, while they mated with the related male in the remaining 9 trials. Females copulated successfully with both males in 9 additional trials. In these trials, females mated with the unrelated male first in 5 traits and with the related first in 4 trials (I don't think it's worthwhile doing a test for only 9 samples given the values). Females were as likely to mate with a second male when they first had mated with the related and the unrelated male (Z_{18} = 1.67, p = 0.09). There was no difference between the number of times that females copulated with the unrelated and related male (Z = 0.86, p = 0.39; Figure. 1a). Furthermore, females copulated first with the larger of the males in 5 trials and the smaller of the two males in 4 trials same as above, and

there was no difference between the number of times that females copulated with the larger and smaller male ($Z = -1.29$, $p = 0.20$). However, females copulated more frequently with the male that was attached to the front leg of the carcass as opposed to the male that was attached to the hind leg ($Z = -2.69$, $p = 0.007$) (Figure. 1b), suggesting that males had a greater mating success when positioned towards the front end of the carcass. There was a non-significant trend for females to mate more frequently with the male that they had encountered first ($Z = 1.91$, $p = 0.057$). In summary, our results provide no evidence for inbreeding avoidance through mate choice when females encounter males simultaneously.

Sequential Mate Encounters

Females copulated successfully with the male that they were presented with in 41 out of 50 trials. Females were as likely to copulate successfully when paired with a related male as when they were paired with an unrelated male ($Z_{48} = 1.09$, $p = 0.28$) (Figure. 2a). Furthermore, there was no significant difference in the latency to copulation with the male between females that were paired with related and unrelated males ($Z_{39} = -0.02$, $p = 0.98$) (Figure. 2b). Thus, our results provide no evidence for inbreeding avoidance through mate choice when females encounter males sequentially.

Discussion

We found that *N. vespilloides* females were as likely to mate with related males as they were with unrelated males regardless of whether they encountered males simultaneously or sequentially. Thus, our study adds to a growing list of studies reporting that females show no mating preference between unrelated and related males (Edly-Wright et al. 2007; Alho et al. 2012; Nichols et al. 2014). We predicted that *N. vespilloides* would show inbreeding avoidance on the basis of Kokko & Ots's (2006) model, which predicts that inbreeding avoidance is more likely to evolve in species with severe inbreeding depression and biparental care. Previous work confirms that *N. vespilloides* suffers from severe inbreeding

239 depression as inbred individuals have lower survival until adulthood and reproduce less
240 successfully as adults than outbred individuals (Mattey et al. 2013), and that both parents of
241 both sexes provide high levels of care for the offspring (Smiseth and Moore 2004; Smiseth et
242 al. 2005). Thus, the lack of inbreeding avoidance through active mate choice in *N.*
243 *vespilloides* may reflect that the evolution of inbreeding avoidance through active mate
244 choice depends on not only on these two conditions, but also on a range of other conditions
245 such as (i) the opportunity costs associated with mating (Kokko & Ots 2006), (ii) the
246 presence of cues that allow females to discriminate between related and unrelated males
247 (Thomas & Simmons 2011), (iii) the costs of inbreeding and/or the risks of inbreeding (Kokko
248 & Ots 2006), and (iv) the presence of alternative mechanisms for inbreeding avoidance
249 (Pusey 1987; Pusey & Wolf 1996).
250 Our finding that females were as likely to mate with related and unrelated males
251 across both experiments provide no evidence that the opportunity costs associated with
252 mating are driving the evolution of inbreeding avoidance in *N. vespilloides*. The model by
253 Kokko & Ots (2006) predicts that females are more likely to avoid mating with related males
254 when they encounter males simultaneously then when they encounter males sequentially.
255 The reason for this is that the opportunity costs should be negligible in the former context
256 because females can simply choose whether to mate with the unrelated or related male. In
257 contrast, these costs could be substantial when females encounter males sequentially
258 because, should the females choose not to mate with a single related male, they run the risk
259 of not encountering an alternative unrelated male, consequently paying a cost in terms of
260 lost mating opportunities. Thus, our results suggest that the presumed difference in the
261 opportunity costs of inbreeding avoidance in our two experiments had no measurable impact
262 on inbreeding avoidance in *N. vespilloides*. Based on this consideration, we find it unlikely
263 that the opportunity costs of inbreeding avoidance are likely to provide an explanation for the
264 lack of inbreeding avoidance in this species.
265 An alternative explanation for the lack of inbreeding avoidance through active mate
266 choice is that females did not have access to cues that they can use to discriminate between

267 related and unrelated male mates. Recent work on a wide range of insects show that
268 females can discriminate between related and unrelated males based on chemical cues,
269 such as cuticular hydrocarbons (CHCs) (Tsutsui 2004; Howard & Blomquist 2005; Weddle et
270 al. 2013). Currently, it is unclear whether females use information on the CHC profiles of
271 males during mate choice in *N. vespilloides*. However, there is good evidence that breeding
272 females use such information to discriminate between their male breeding partner and non-
273 breeding male intruders based on differences in their CHC profiles (Müller et al. 2003;
274 Steiger et al. 2007). Thus, given this evidence that females use CHCs to discriminate
275 between males, and that CHCs play a key role in kin discrimination in many insects, it seems
276 likely that females have the ability to assess the genetic similarity of different males based
277 on variation in CHC profiles also in *N. vespilloides*. Nevertheless, this suggestion needs to
278 be substantiated by empirical evidence, and to this end, there is now a need for further work
279 to investigate whether CHC profiles correlate with genetic similarity and whether females
280 preferentially mate with males that have a more dissimilar CHC profile to their own profile.
281 A third explanation for our negative results is that there is selection for inbreeding
282 tolerance in *N. vespilloides* because the costs of inbreeding and/or the risks of inbreeding
283 are relatively low (Kokko & Ots 2006). Previous studies show that inbreeding exerts
284 substantial fitness costs throughout the entire life cycle in *N. vespilloides*. For example,
285 inbred offspring suffer a reduction in survival during the juvenile stage by 11% compared to
286 that of outbred offspring (Mattey et al. 2013). Furthermore, inbred adults suffer a reduction in
287 breeding success by 22% compared to that of outbred parents (Mattey et al. 2013). Thus, it
288 seems unlikely that there is selection for inbreeding tolerance because the costs of
289 inbreeding are low. Currently, little is know about the risks of inbreeding in *N. vespilloides*.
290 However, the woodland areas in which we collected the beetles used in these experiments
291 support a relatively large population (P.T. Smiseth, unpublished data). Given that
292 *Nicrophorus* beetles can locate a carcass from several kilometres away (Petruška 1975), it
293 seems likely that they also disperse widely. As a consequence, the risks of inbreeding might
294 be relatively low in this species. Indeed, the high costs of inbreeding (Mattey et al. 2013)

might reflect that there is no history of purging due to inbreeding in this population, as would be expected if the risks of inbreeding are low.

A final explanation for the lack of inbreeding avoidance through active mate choice is that selection has favoured inbreeding avoidance through other mechanisms, such as sex-biased dispersal and polyandry coupled with post-copulatory female choice (Pusey 1987; Pusey & Wolf 1996). Currently, there is no information on sex-biased dispersal in *N. vespilloides*, and it is therefore unclear whether inbreeding avoidance might occur via sex-biased dispersal. However, *Nicrophorus* beetles are known to locate a carcass from several kilometres away (Petruška 1975), suggesting that they have a high potential for dispersal. There is good evidence for polyandry in *N. vespilloides*. Firstly, females mate with pheromone-emitting males in the absence of a carcass and store sperm from such mating until they find a suitable carcass (Bartlett 1987; Eggert 1992). Secondly, when females find a suitable carcass, they often also engage in extra-pair copulations with satellite males (Pettinger et al. 2011). Thus, polyandry might provide an effective mechanism for inbreeding avoidance if coupled with post-copulatory mate choice as in the field cricket *Teleogryllus* (Tregenza & Wedell 2000; Bretman et al. 2009) and guppies *Poecilia reticulata* (Fitzpatrick & Evans 2014). We encourage future work on to examine the role of post-copulatory inbreeding avoidance and sex-biased dispersal in this species.

We found no evidence that females were more likely to mate with the larger of the two males. Our results contradict those of Beeler et al. (2002), who found that females of the closely related *N. orbicollis* preferentially mated with larger males. There are several possible explanations for the contrasting findings of the two studies, including differences in experimental design and differences between the two study species. Firstly, the females in our experiment were placed next to the two males that had been tethered to opposite ends of the carcass. In contrast, the females in the experiment by Beeler et al. (2002) were placed in an olfactometer and could choose between the pheromones produced by two males. Thus, the different results of the two experiments might reflect that females in our experiment had access to a range of olfactory, tactile and behavioural cues, while females in

323 the experiment by Beeler et al. (2002) only had access to volatile pheromones. Secondly,
324 our study was conducted on *N. vespilloides*, while the study by Beeler et al. (2002) was
325 conducted on *N. orbicollis*. Potentially, there might be differences in female mating
326 preferences between these two species.
327 Finally, we found that females were more likely to mate with the male attached to front
328 leg than the male attached to the hind leg. We are unaware of prior studies showing that
329 females preferentially mate with males at different positions on the carcass. One potential
330 explanation for this finding is that females preferentially mate with males at the front end
331 because such males somehow are superior to males at the rear end of the carcass. This
332 explanation seems unlikely, as the dominant male usually drives his rivals away from the
333 carcass (Otronen 1988). Alternatively, our results are likely to reflect that females spent a
334 larger proportion of time performing preparation behaviour at the front end of the carcass
335 (personal observation), and so females encountered males positioned at the head end more
336 frequently, allowing the males to engage in more matings with the female. non-significant
337 effect. Based on this finding, we suggest that, whenever a male locates a carcass before the
338 female, he should preferentially spend more time towards the head end of the carcass as
339 this would speed up the time until he detects the presence of a female.
340 To conclude, we find no evidence for inbreeding avoidance through active mate choice
341 in *N. vespilloides* despite that this species suffers from severe inbreeding depression (Mattey
342 et al. 2013) and engages in biparental care (Smiseth & Moore 2004; Smiseth et al. 2005). To
343 our knowledge, our study is the first to use both simultaneous and sequential mate choice
344 designs in a study of inbreeding avoidance. We note that a recent meta-analysis on female
345 choice in contexts other than inbreeding avoidance found that mating preferences were
346 significantly stronger when females were presented with a simultaneous choice between
347 multiple male partners ('choice designs') than when females were presented with a
348 sequential choice between single males ('no choice designs'; Dougherty & Shuker 2015).
349 These results might reflect females can make a direct comparison between related and
350 unrelated mates when they encounter males simultaneously, while females must make a

decision about whether or not to mate with a single male based on a template of a preferred partner when they encounter males sequentially. As a consequence, females may be able to detect smaller differences between males when females encounter males simultaneously (Wagner 1998). Based, on these considerations, we suggest that studies on the role of active mate choice as a mechanism for inbreeding avoidance need to consider the implications of different mate choice designs. Thus, we encourage the use of experimental designs where females encounter males both simultaneously (i.e., choice designs) and sequentially (i.e., no choice designs) in future studies on other species.

Acknowledgments

We thank the City of Edinburgh Council Natural Heritage Service for permission to collect beetles at Corstorphine Hill and the Hermitage of Braid, and Daniel Rozen at the University of Leiden, Nick Royle at the University of Exeter and Rebecca Kilner at the University of Cambridge for supplying beetles from Jodrell Bank, Manchester, Kennel Vale, Cornwall and Madingley Woods, Cambridge, respectively. We thank Ronnie Mooney and Clare Andrews for assistance in maintaining the laboratory population. We thank Marie Herberstein, Beate Nicolai, Jane Reid and Craig Walling for valuable comments on the manuscript. The research was funded by a studentship from the Natural Environment Research Council to S.N.M. The authors confirm that they have no conflict of interest to declare.

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466

Figure Legends

Figure 1. Inbreeding avoidance by simultaneous mate choice in *Nicrophorus vespilloides*. (a) The number of times a female mated with either a related (white bars) or an unrelated male (grey bars), and (b) the number of times a female mated with a male tethered to the front leg (white bars) or the hind leg (grey bars) of the carcass (means ± 1 SE).

Figure 2. Inbreeding avoidance through sequential mate choice in *Nicrophorus vespilloides*. (a) The number of females that mated with either an unrelated (white bars) or a related male (grey bars), and (b) the copulation latency for females that mated successfully with an unrelated (white bars) or a related male (grey bars) (mean ± 1 SE).